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Chemistry and Bioactivity of An Artificial Adenosylpeptide B12 Cofactor

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Coenzyme B_{12} dependent reactions play a key role in the metabolism of humans and many other life forms. We describe a bio-inspired semi-artificial adenosylpeptide B_{12} that behaves as cofactor in B_{12}-dependent enzymatic reactions of glutamate mutase from Clostridium cochlearium and of ribonucleotide reductase from Lactobacillus delbrueckii. It is demonstrated that the peptide backbone influences the chemical properties of the artificial adenosylcobalamin cofactor and modulates the bioactivity in vitro and in vivo. In addition, inhibition of bacterial growth of L. delbrueckii is demonstrated with an IC50 value of 2 μM after ten hours, providing a potentially powerful approach for the development of antibacterial and antiproliferative compounds with a novel mode-of-action.

Organometallic compounds have gained considerable attention for medical purposes.[1-3] Rationally designed metal complexes can competitively bind to a protein-binding pocket and effectively inhibit enzymatic function.[4-8]

The development of Ru^{II}-metal complexes as potent and selective kinase inhibitors are probably the most prominent current examples.[9] In contrast to fully synthetic enzyme inhibitors, rather little attention is paid to the development of structurally perfect, but catalytically inactive surrogates of essential cofactor catalysts. The cofactors methylcobalamin (MetCbl) and adenosylcobalamin (AdoCbl) have been considered for long time the only organometallic compounds in biological systems and they are essential for the metabolism of humans and many other life-forms.[10] MetCbl-dependent methionine synthase (MetH) is the only human enzyme able to convert 5-methyltetrahydrofolate to unsubstituted tetrahydrofolate required for purine and pyrimidine biosyntheses in humans, whereas AdoCbl-dependent ribonucleotide reductase (RNR) is essential for DNA replication in certain microorganisms.[11]

In our efforts to develop anti-vitamins as inhibitors of vitamin B_{12}-dependent enzymes,[12-14] we report the coordination chemistry and bioactivity of an organometallic peptide B_{12} cofactor (**1**$^+$, Scheme 1). We envisaged that an artificial biomimetic peptide backbone replacing the α-ribazole linker at the "lower" face of the corrin macrocycle would strongly modulate the 'base on/ base off' equilibrium of the artificial cofactor and thereby affect enzymatic catalysis in both, the 'base on' as well as 'base off/ histidine on' constitutions (Figure 1).[14-17] Adenosylpeptide B_{12} (Ado-pB_{12}; **1**$^+$) was synthesized from pB_{12} (**3**$^+$)[14] and 5'-chloro-5'-deoxyadenosine under reductive conditions (Scheme 2),[18-20] isolated by preparative reverse phase C18 chromatography and analyzed by UV-Vis, ^{1}H-NMR and MS spectrometry.

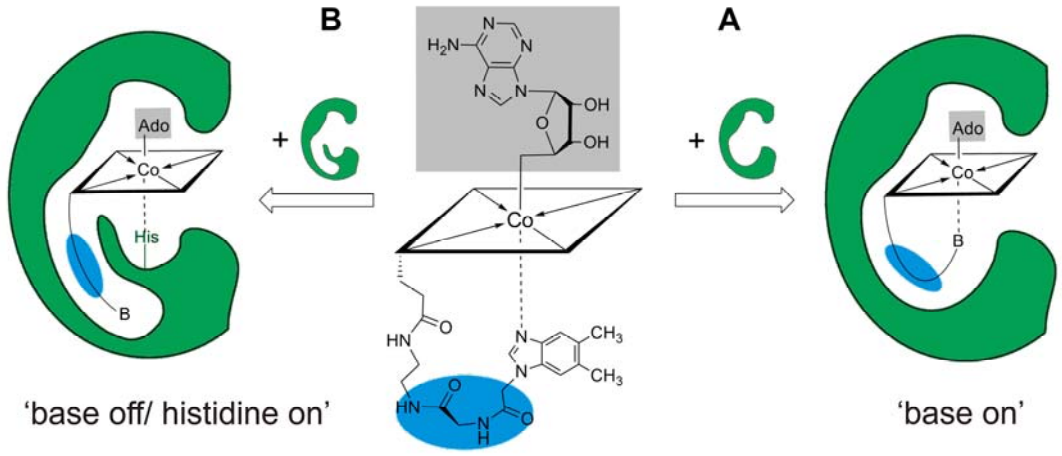


Figure 1. Schematic model for the binding of the semi-artificial adenosylpeptide B_{12} cofactor to enzymes in either the 'base on' (A) or 'base off/ histidine on' constitution (B). The structurally modified backbone and the unaltered adenosyl moiety of the cofactor are shown in blue and grey and the enzymes are depicted green.

In the high-resolution mass spectrum of $\mathbf{1}^+$, a signal at *m/z* 734.34579 (m/z_{calc} 734.34496) corresponding to $[\mathbf{1}^+ + H]^{2+}$ ions with the molecular formula of $C_{70}H_{97}CoN_{20}O_{12}$ was observed.

The "unique" UV-Vis spectrum of **1** (**1**: $\mathbf{1}^+$ x CF_3COO^-; Figure 2) with one broad absorption for the α/β-bands is comparable to the absorption spectra of **2** in the 'base on' form.[21]

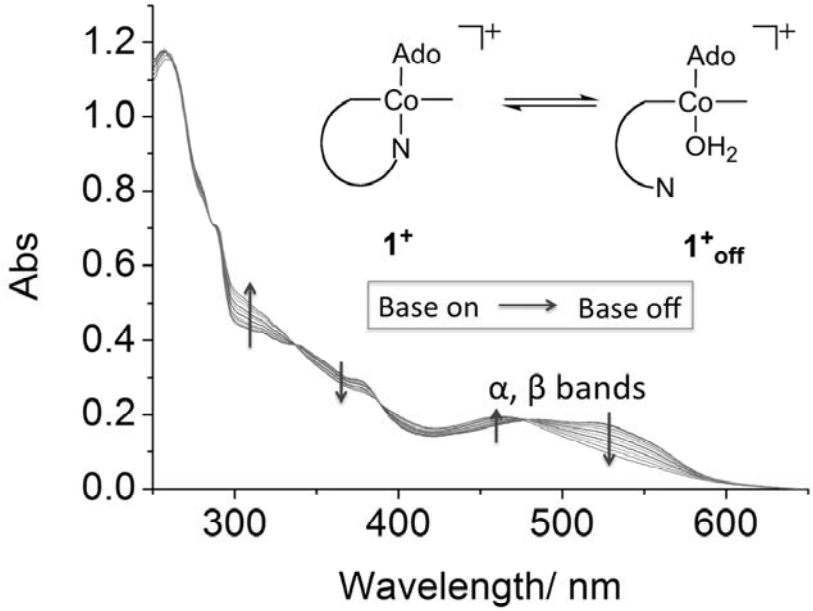
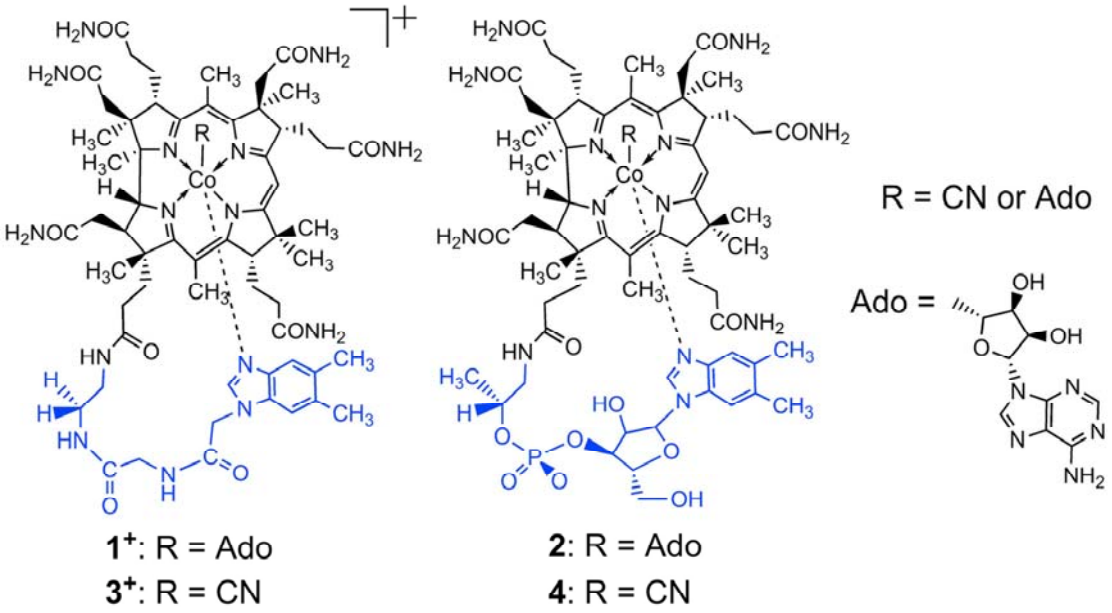


Figure 2. Temperature dependent UV-Vis spectra of **1** (18 μM; Britton-Robinson buffer[18], pH 7.5; T= 0-90°C). The absorption values at 517 nm have been used for calculating T_{mid} ($[\mathbf{1}^+]/[\mathbf{1}^+_{off}] = 1$) = 65°C by Boltzmann simulation (Figure S2).

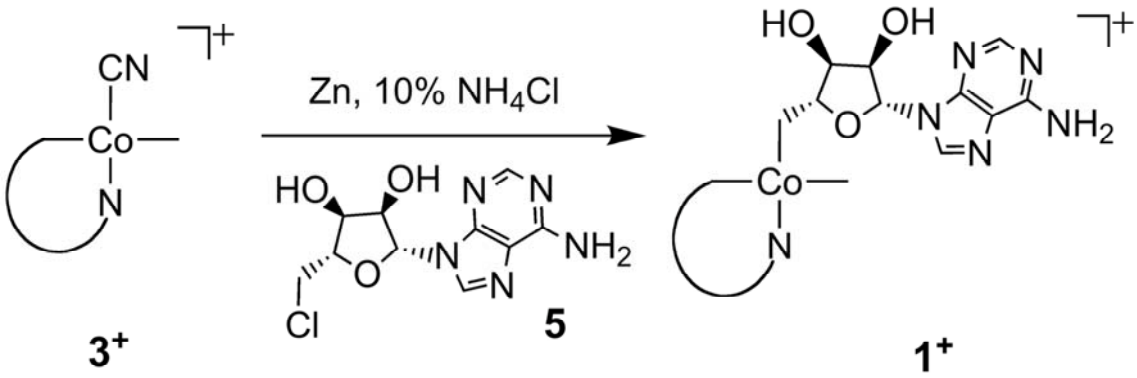
Spectrophotometric pH titrations of **1** revealed a $pK_{base\ off}$ value of 4.4 (Figure S1),[18] indicating that the 'base on' form is eight times less favored compared to **2** ($pK_{base\ off}$ = 3.5).[22] Dissociation of the 5,6-dimethylbenzimidazole (Dmbz) base from the Co^{III} centre was also observed with temperature dependent UV-Vis experiments at pH 7.5 (Figure 2) indicating a Gibbs energy of -6.6 kJ/mol as outlined in the Supporting Information.[23] This value is slightly higher than that of **2** (ΔG_0 = -10.8 kJ/mol[24]). The corresponding enthalpy (ΔH_0) and entropy (ΔS_0) values are -49.7 kJ/mol and -147 J/Kmol, respectively.[18] A correlation between the $pK_{base\ off}$ and the ΔH_0 and ΔS_0 values is observed (Figure 3).

This trend is underscored by comparison with the thermodynamic values of other alkylcobalamins having either different carbon-based ligands (**5-8**) or a modified α-ribazole backbone (**9**) (for structures of **5-9** see Figure S3).[18, 23, 25-27] The impact of the malfunctioning 'base on/ base off' trigger of the artificial cofactor on the bioactivity was studied with the microorganism *L. delbrueckii* and the enzyme glutamate mutase from *Clostridium cochlearium*. The former requires 'base on' adenosylcobalamin (**2**) as cofactor for RNR as the only B_{12}-dependent enzyme (Figure 1 A),[28] whereas the Dmbz base of **2** is substituted with a histidine side chain

of the protein ('base off/ histidine on') in the active site of glutamate mutase (Figure 1 B).[29] The 'loop' consisting of propanolamine, phosphate, ribose and Dmbz sits in a pocket of component S. AdoCbl-dependent glutamate mutase triggers the reversible radical rearrangement between (*S*)-glutamate and (2*S*,3*S*)-3-methylaspartate (Table 1).[30]



Scheme 1. Structural formula of Ado-pB12 (**1**$^+$), AdoCbl (**2**), pB_{12} (**3**$^+$), and B_{12} (**4**). The artificial and natural loop structures are shown in blue.



Scheme 2. Synthesis of the artificial cofactor. Ado-pB_{12} (**1**$^+$) was synthesized from **3**$^+$. The counter-ions are CF_3COO^-.[18]

The activity of glutamate mutase was determined in a standard coupled assay at two different ratios of the protein components GlmS and GlmE (S:E = 2 or 14) with either **1** or **2** as cofactor.[31] Similar apparent Km values for **1** and **2** suggest equal affinities of the cofactors to the enzyme and indicate that both cofactors were able to reconstitute hologlutamate mutase from GlmS and E. The catalytic efficiency (kcat/Km, $s^{-1}M^{-1}$) of **1**, however, was approximately 10 times reduced as compared to

that of **2** (Table 1). This is probably caused by a slightly tilted or kinked binding of the dissociated, artificial peptide loop to subunit S of the protein.[32]

B_{12}-dependent cell growth studies with *L. delbrueckii* were conducted to gain insights into the in vivo activity of pB_{12} (**3**). A strongly reduced, yet residual concentration dependent bioactivity of **3** was observed (Figure 4, lower part).

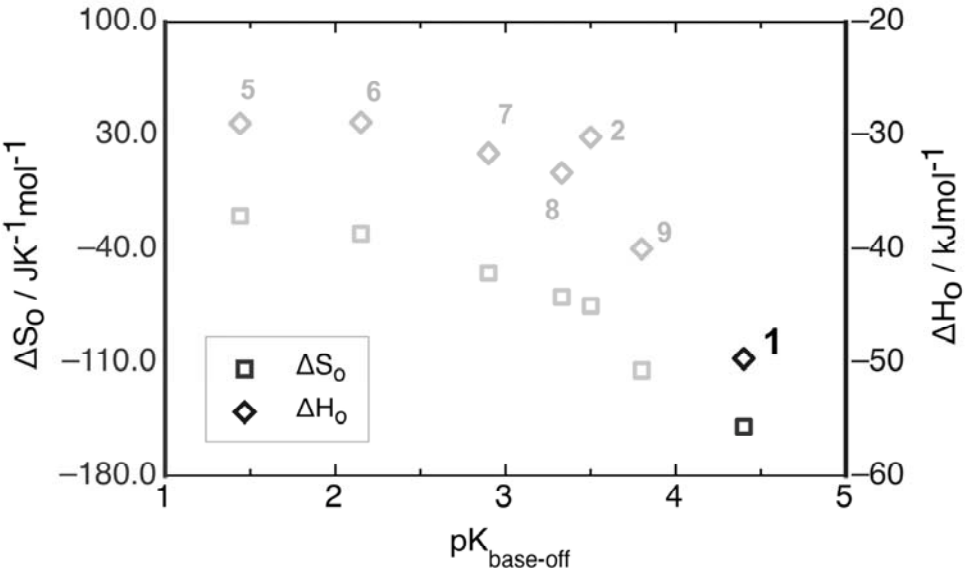


Figure 3. Plot of the $pK_{base\ off}$ values of various alkyl-cobalamins vs their entropy and enthalpy values.[13, 17, 18, 20, 21]

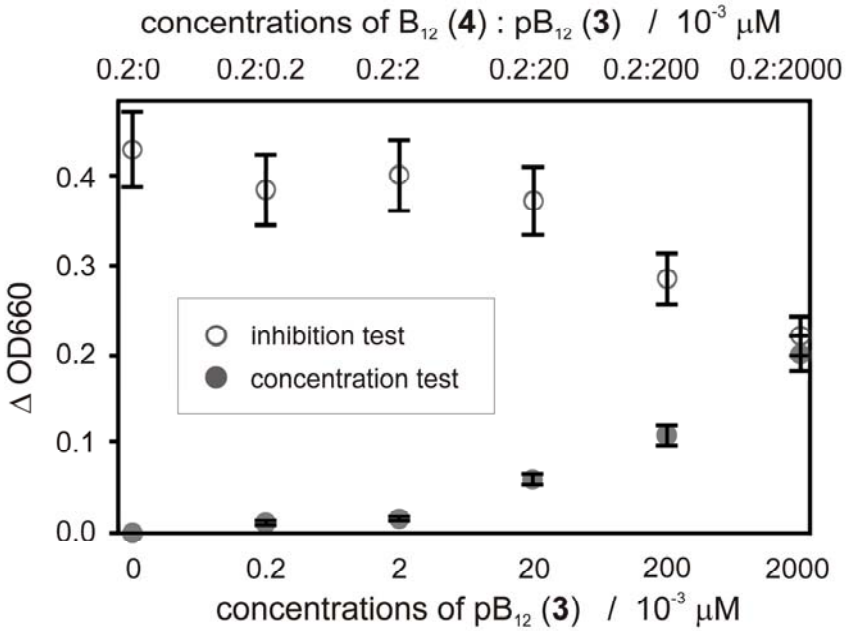
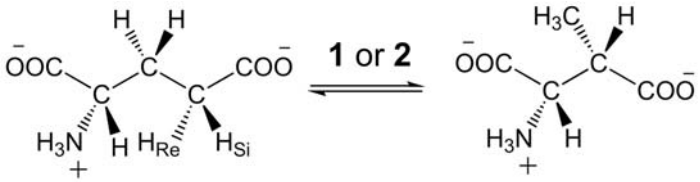


Figure 4. Solid circles: Concentration dependence of the bioactivity of pB_{12} (**3**) at 37°C (n=3) after 10 hours. Hollow circles: Inhibition of the bioactivity of vitamin B_{12} (**4**, 0.2 nM) by pB_{12} (**3**, concentrations ranging from 0.2 nM to 2 µM) at 37°C (n=3) after 10 hours (10 hours = maximum growth rate in the exponential phase; Δ OD660: the net absorption of the samples at 660 nm compared to the blank measurement).

This behaviour gives evidence that the microorganism still recognizes, internalizes and metabolizes $\mathbf{3}^+$ into its organometallic analog $\mathbf{1}^+$.[13] The artificial cofactor is still catalytically active in the 'base on' constitution with an apparent Km of ca. 2 μM (Figure 4).

Competition experiments with B_{12} (**4**, 0.25 ng/ml) and increasing amounts of **3** (1-10000 eq.) revealed an IC50 value of 2 μM after 10 h. Under these conditions, bacterial growth depends apparently solely on the reduced bioactivity of **3** (Figure 4, upper part *versus* lower part). The degree of inhibition is time dependent and decreases from 60% after 6 h (onset exponential phase) to around 20% after 24 h (stationary phase), most likely due to the replacement of $\mathbf{1}^+$ with **2** (Figure S4).[18]

Table 1. Kinetic constants of **1** and **2** in the reaction catalyzed by partially purified glutamate mutase from Clostridium cochlearium at different ratios of components S and E. The rearrangement is initiated by abstraction of H_{Si} by the 5'-deoxyadenosyl radical from C4 of glutamate.



	apparent K_m [μM]		k_{cat} [s^{-1}]		k_{cat}/K_m x 10^{-6} [$s^{-1}M^{-1}$]	
GlmS:GlmE	14	2	14	2	14	2
1	0.35±0.05	1.07±0.04	0.09±0.01	0.12±0.01	0.26	0.11
2	0.52±0.06	1.12±0.04	1.24±0.36	1.24±0.36	2.38	1.10

Although the two systems are not directly comparable, we attribute the apparent lowered activity of $\mathbf{3}^+$ in vivo compared to the in vitro activity of $\mathbf{1}^+$ to the complex (i) bacterial uptake and (ii) metabolism to the organometallic cofactor $\mathbf{1}^+$. Intuitively, it is reasonable that (iii) the requirement of a 'base on' constitution of the cofactor in RNR – *in contrast to the 'base off' constitution in glutamate mutase* – affects enzymatic much more strongly. The Dmbz base of the peptide backbone is coordinated to the cobalt center and directly influences the *trans*-located cobalt-carbon bond of the artificial cofactor.

Some modified B_{12} derivatives have already been tested as anti-vitamins in pioneering studies[33, 34] or have been applied as probes in enzymatic reactions.[32, 35-38]

Here we used for the first time an artificial cofactor with a biomimetic peptide backbone, studied its intramolecular coordination chemistry and demonstrated reduced catalytic efficiencies in both, the 'base on' and 'base off/ histidine on' constitutions.

The inhibitory potential of peptide B_{12}s is useful for the development of novel antibacterial and antiproliferative agents.

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